Plasma Nitric Oxide Concentrations in Broilers After Intravenous Injections of Lipopolysaccharide or Microparticles


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ABSTRACT Nitric oxide is a potent vasodilator synthesized from L-Arg by NO synthase (NOS). Constitutive NOS in endothelial cells (eNOS) produces transient bursts of NO in low but physiologically effective levels. Activated monocytes and macrophages express inducible NOS (iNOS), which produces copious quantities of NO. Previous studies showed that NO attenuates pulmonary hypertensive responses induced by i.v. injections of lipopolysaccharide (LPS) or cellulose microparticles (MP). The present study determined whether changes in plasma NO concentrations could be used to assess the time course of NO production in response to LPS or MP injections. Broilers were injected i.v. with 1 mL of PBS (control), 1 mL of LPS (1 mg/mL), or 0.4 mL of MP (0.02 g/mL). Plasma samples were collected from 10 different broilers per group at 15, 30, 45, and 60 min and at 2, 3, 4, 5, 6, 8, 10, and 12 h postinjection. Total plasma NO concentrations were analyzed by nitrate + nitrite assay. After PBS or MP injection, plasma NO levels did not change throughout the 12-h period. Nitric oxide measured in the plasma increased in LPS-injected broilers from 4.8 ± 0.8 μM at 15 min to 46.6 ± 5.7 μM by 4 h postinjection, reached peak levels of 85.1 ± 10.6 μM at 5 h, and returned to baseline levels similar to PBS-injected broilers by 12 h postinjection. We conclude that LPS triggered widespread iNOS expression by circulating monocytes and macrophages, resulting in copious NO production as reflected by significant increases in total plasma NO. Proportionally few monocytes and macrophages responded to MP entrapped in pulmonary arterioles. Consequently, NO produced by iNOS in activated leukocytes or by eNOS in the pulmonary vasculature had a minimal impact on total plasma NO. Total plasma NO from broilers did reflect the time course of massive iNOS activation in response to LPS, but biologically relevant quantities of NO produced by iNOS and eNOS activated during the local inflammatory response to entrapped MPs were too low to affect total plasma NO concentrations.

Key words: broiler, nitric oxide, lipopolysaccharide, cellulose microparticle

INTRODUCTION Pulmonary hypertension syndrome (PHS, ascites syndrome) develops in broilers when an elevated pulmonary vascular resistance forces the right ventricle to increase the pulmonary arterial pressure (PAP) to propel the cardiac output through the lungs (Wideman and French, 1999; Wideman et al., 2001, 2004). The elevated pulmonary vascular resistance in PHS-susceptible broilers reflects an anatomically inadequate pulmonary vascular capacity combined with vasoconstriction attributable to serotonin and perhaps thromboxane or endothelin-1. The potent vasodilator NO directly reduces the pulmonary vascular resistance and modulates the release of serotonin, thromboxane, and endothelin-1 (Wideman et al., 2001, 2004, 2005, 2006; Wideman and Chapman, 2004; Bowen et al., 2006a,b; Chapman and Wideman, 2006). Nitric oxide is produced from L-Arg by the enzyme NO synthase (NOS). Endothelial NOS (eNOS, or NOS-3) is constitutively expressed by endothelial cells lining the vascular lumen. When increased blood flow and pressure exert increased shear stress on the endothelium, eNOS transiently produces NO in low but biologically effective nanomolar bursts (Albrecht et al., 2003; Martinez-Lemus et al., 2003; Deem, 2004). Inducible NOS (iNOS, or NOS-2) is expressed by activated monocytes and macrophages (Pulido et al., 2000; Dil and Qureshi, 2002a,b) and generates copious, sustained quantities of NO at micromolar levels due to the essentially irreversible binding of Ca2+ to calmodulin associated with the iNOS enzyme (Andrew and Mayer, 1999; Hecker et al., 1999).

Gram-negative bacteria and lipopolysaccharide (LPS, endotoxin), a cell wall component of gram-negative bacteria, are common aerosol contaminants within broiler houses that are known to trigger pulmonary vasoconstriction, pulmonary hypertension, and PHS (Tottori et al., 1997; Yamaguchi et al., 2000; Wideman et al., 2001, 2004;
Wang et al., 2002a, 2003a,b; Bakutis et al., 2004). When LPS is injected i.v., the PAP typically begins to increase within 15 to 20 min, reaches peak levels within 25 to 35 min, and then recedes toward the preinjection baseline pressure by 60 min postinjection (Wideman et al., 2001, 2004; Wang et al., 2002a, 2003a). Nitric oxide clearly modulates the pulmonary hypertensive response to LPS. Administering Nω-nitro-L-Arg methyl ester (L-NAME), a competitive inhibitor of both eNOS and iNOS, increases the peak PAP attained in response to LPS and reverses the postpeak decline in PAP (Wideman and Chapman, 2004; Bowen et al., 2006b). Pretreatment with aminoguanidine, a specific inhibitor of iNOS, does not alter the acute (within 60 min) pulmonary hypertensive response to LPS, suggesting that NO derived from eNOS modulates acute responses to LPS, whereas NO produced by iNOS expressed by activated monocytes and macrophages may modulate the more chronic (>90 min) responses (Bowen et al., 2006a,b; Chapman and Wideman, 2006).

Intravenous cellulose microparticle (MP) injections also can be used to induce pulmonary hypertension and PHS in broilers. The MP become entrapped in the pulmonary vasculature where they physically occlude terminal pulmonary arterioles and trigger focal intrapulmonary inflammation. The MP are rapidly (within minutes) surrounded by activated monocytes and thrombocytes, followed thereafter by the formation of mononuclear cell aggregates in the perivascular area of the MP-occluded vessels that persist for 2 wk until the MP are cleared from the lungs (Wideman and Erf, 2002; Wideman et al., 2002, 2005, 2006; Wang et al., 2003b). The pulmonary hypertensive response and subsequent mortality triggered by i.v. MP injections were doubled when broilers were pretreated with L-NAME, whereas pretreatment with aminoguanidine did not consistently alter the pulmonary hypertensive response to MP injections (Wideman et al., 2005, 2006). These observations suggest that the acute responses to i.v. MP injections are modulated primarily by NO produced by eNOS. Increased iNOS expression has been detected in the lung parenchyma within 24 h following i.v. MP injection (Hamal et al., 2006), but a chronic modulatory role for NO produced by iNOS remains to be demonstrated in MP-injected broilers.

The present study was conducted to measure plasma NO concentrations during the acute (15 to 120 min) and more chronic (2 to 12 h) responses to i.v. injections of LPS or MP. Based on the results of a previous study, it was our hypothesis that low but biologically effective nanomolar levels of NO produced by eNOS during the acute responses to LPS and MP may not be detectable in the plasma, whereas the copious micromolar quantities of NO generated by iNOS should significantly increase total plasma NO concentrations (Chapman and Wideman, 2006). Determining the in vivo time course of maximal iNOS activation also was needed to facilitate future evaluations of the potential modulatory impact of NO produced by iNOS in broilers responding to LPS and i.v. MP injections.

MATERIALS AND METHODS

Broiler Management

Male broilers were transported on the day after hatch (d 1: January 4, 2006) from a commercial hatchery to the Poultry Environmental Research Laboratory at the University of Arkansas Poultry Research Farm. The chicks were wing-banded and placed on fresh litter in environmental chambers (8 m² of floor space). Chicks were reared at 33°C on d 1 to 4, 29°C on d 5 to 6, 27°C on d 7 to 10, 24°C on d 11 to 15, and 22°C through d 35 when the experiment was terminated. The chicks were exposed to 24 h of light for d 1 to 4 and 16L:8D thereafter. A corn soybean meal starter ration was provided ad libitum throughout the experiment that was formulated to meet or exceed NRC (1994; 22.7% CP, 3,059 kcal of ME/kg, 1.5% Arg, and 1.43% Lys) standards for all ingredients. The diet was provided as crumbles for the first 1 to 2 wk and then in pellet form thereafter. Water was provided ad libitum via nipple-type waterers.

MP, LPS, and PBS Injections

Five-week-old broilers were injected i.v. with cellulose MP, LPS, or PBS (vehicle control). Microgranular CM-32 ion exchange cellulose particles (Fisher Scientific, St. Louis, MO) were suspended in heparinized saline (Sigma Chemical Co., St. Louis, MO) at 0.02 g/mL (150 units of ammonium heparin/mL of 0.9% NaCl). One-milliliter syringes with 23-gauge needles were used to inject 120 broilers with 0.4 mL of the MP suspension (Wideman and Erf, 2002; Wideman et al., 2002, 2003). Lipopolysaccharide (Sigma Chemical Co.) from Salmonella Typhimurium was diluted in PBS at 1 mg/mL, and 1 mL was injected into 122 broilers. One milliliter of PBS (vehicle) was injected into 100 broilers to establish baseline plasma NO levels. The broilers were returned to their environmental chambers, where they had ad libitum access to full feed and water.

Three-milliliter blood samples were collected at 15, 30, 45, and 60 min and at 2, 3, 4, 5, 6, 8, 10, and 12 h postinjection from MP-, LPS-, and PBS-injected broilers (average weight after injection: 1,931 ± 19 g, 1,895 ± 26 g, and 1,936 ± 43 g, respectively; mean ± SEM; P = 0.544). Ten different broilers were sampled per treatment at each time interval. Blood was collected with 3-mL syringes containing 0.1 mL of EDTA (0.9% saline solution with 50 mg of EDTA/mL) that was used as an anticoagulant. The blood samples were divided into 1.5-mL microcentrifuge tubes and centrifuged for 1 min at 150 × g. Plasma was separated from the cell pellet and stored in 1.5-mL microcentrifuge tubes at −20°C.

Plasma NO Assay

Nitrate (NO₃⁻) + nitrite (NO₂⁻) were measured in the plasma using the Cayman Chemical Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Co., Ann Arbor,
MI). This assay converts NO\textsuperscript{3−} to NO\textsuperscript{2−} with nitrate reductase, and total NO is measured as total nitrite using the Griess reagent. Because NO is a free radical, it is oxidized quickly into NO\textsuperscript{2−} and NO\textsuperscript{3−} in aqueous solutions and blood, allowing the measurement of total nitrite to equal total NO production (Hampl and Herget, 2000). The plasma was analyzed according to kit instructions. The plasma was thawed and filtered through Microcon YM-30 Centrifugal Filters (Millipore Corporation, Bedford, MA), which were presoaked with ultrapure water and centrifuged to remove any background absorbance attributable to hemoglobin in the plasma. Ethylenediaminetetraacetate was used as the anticoagulant to prevent precipitation that may occur when heparinized plasma is mixed with the Griess reagent. The assays were analyzed in a 96-well plate using a BioTek Powerwave X340 (Bio-Tek Instruments Inc., Highland Park, VT) at a wavelength of 540 nm. A nitrite standard curve was generated in each plate to determine total nitrite (\(\mu M\)).

**Data Analysis**

Mean plasma nitrite concentrations were determined within MP, LPS, and PBS injection groups over all time points using SigmaStat (Jandel Scientific, 1994) 1-way ANOVA. Plasma nitrite concentrations were also analyzed at each time point for differences among injection groups using SigmaStat Tukey’s 1-way ANOVA. Differences between means were considered significant when \(P < 0.05\).

**RESULTS AND DISCUSSION**

Intravenous injections of LPS and MP are known to elicit acute increases in PAP that gradually subside toward preinjection levels during the ensuing 60- to 90-min postinjection. Previous studies demonstrated that administering L-NAME, a competitive inhibitor of both eNOS and iNOS, increased the amplitude and duration of the pulmonary hypertensive responses to LPS and MP. Accordingly, during the acute postinjection interval, NO appears to prevent the development of maximal pulmonary hypertensive responses by acting as a potent pulmonary vasodilator and by modulating (inhibiting) the release of key vasoconstrictors (Wideman et al., 2004, 2007). The present study was designed to determine whether changes in plasma NO concentrations can be used to assess the time course of NOS activation and NO production during the acute (15 to 120 min) and more chronic (2 to 12 h) responses to i.v. injections of MP and LPS. The results shown in Figure 1 demonstrate that i.v. injections of PBS and MP did not increase plasma NO concentrations for up to 12 h postinjection (15-min value compared with subsequent values within a group using 1-way ANOVA). The LPS injection also did not elicit increases in plasma NO for up to 120 min postinjection, but thereafter, plasma NO concentrations (mean \(\pm\) SEM) increased above \((P < 0.001)\) the baseline levels (4.8 \(\pm\) 0.8 \(\mu M\)) by 4 h postinjection (46.6 \(\pm\) 5.7 \(\mu M\)), peaked at 5 h (85.1 \(\pm\) 10.6 \(\mu M\)), and returned toward baseline levels by 12 h postinjection. For reasons that remain to be determined, the plasma NO concentrations in the PBS group were higher than in the LPS group at 60 min postinjection, and higher than in the MP group at 60 min, 2 h, and 12 h postinjection (Figure 1).

Previous physiological and pharmacological studies indicated that eNOS primarily modulates the acute (15 to 120 min) pulmonary hypertensive responses to MP and LPS (Bowen et al., 2006a,b; Chapman and Wideman, 2006). Activated eNOS releases NO locally in low but
biologically effective quantities that are diluted rapidly in the extracellular fluid, bind to hemoglobin, exhaled in the form of NO gas, and are rapidly cleared from the blood by the kidneys (Wang et al., 2002b; Chapman and Wideman, 2006). The present results confirm that the low levels of NO produced during the acute responses to LPS and MP did not cause significant NO accumulation in the plasma. In contrast, activated iNOS generates copious quantities of NO that can significantly increase total plasma NO concentrations (Chapman and Wideman, 2006). Accordingly, the large increase in plasma NO detected 4 to 8 h after injecting LPS in the present study appears to reflect system-wide expression and massive activation of iNOS in circulating monocytes and macrophages (Hussain and Qureshi, 1997; Bowen et al., 2006b). Evidently, proportionally fewer monocytes and macrophages respond to entrapped MP, resulting in lower concentrations of NO (focal response) in the plasma and allowing rapid dilution from the local fluids, thereby preventing measurable increases in plasma NO concentrations during the 12 h following MP injection (Figure 1; Wang et al., 2003b; Hamal et al., 2006). Mononuclear leukocytes previously have been demonstrated to aggregate within minutes after MP become entrapped in the pulmonary vasculature, forming a perivascular granuloma-like aggregate by 48 h postinjection (Wideman and Erf, 2002; Wideman et al., 2002; Wang et al., 2003b). Inducible NOS expression also has been shown to increase during the 24 h following an i.v. MP injection (Hamal et al., 2006). Further studies are needed to determine whether iNOS activation and a corresponding increase in plasma NO concentration can be detected 12 to 48 h post MP injection.

In conclusion, eNOS activation was not detected by measuring NO in the plasma during the acute response to LPS or MP injections, presumably because the low concentrations of NO derived from eNOS were readily diluted and dissipated. Lipopolysaccharide induced a system-wide activation of circulating monocytes, resulting in a multifold increase in total plasma NO concentrations. Changes in plasma NO concentrations were not detected following MP injections, presumably because iNOS was focally activated in proportionally few responding mononuclear leukocytes, or additional time was required to upregulate iNOS expression.

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REFERENCES


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